NEW NON-SPECIFIC QUADRUPLE AND TRIPLE STAINING METHODS FOR LIGHT MICROSCOPY

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(Received December 4, 1972)

For the routine works with light microscope, double staining methods have been usually employed. Only several triple staining techniques are widely known for pathological purposes. These are enumerated as follows: Mallory's acid fuchsin-anilin blue-orange G method; Mallory-Heidenhain's azan staining method (azocarmin-anilin blue-orange G); Masson's trichrome method (iron hematoxylin-acid fuchsin-anilin blue or light green); Goldner's modification of Masson's method (Weigert's hematoxylin-ponceau-acid fuchsin-orange G or light green); Dominici's method (toluidine blue-eosin-orange G); PAP method and PAM method.

These triple staining methods are non-specific, and devised for the pathological analization of tissue elements. These methods give excellent and beautiful results, but are unexceptionally complicated in procedures and give very indefinite results in colour tones. It takes a long experience to obtain the standard permanent preparations. Some of them apt to fade in a very short period as Dominici's method for example. For the diagnosis of cancer *in situ* of uterus and prostate gland, Papanicolaou's method for smear materials is famous and has been adopted as the standard technique.

The present author's works are aiming at the pathological analysis of tissue elements, especially suitable for the malignant alteration in liver. The works consist of quadruple and triple staining methods as follows.

Methods

A. Quadruple staining method

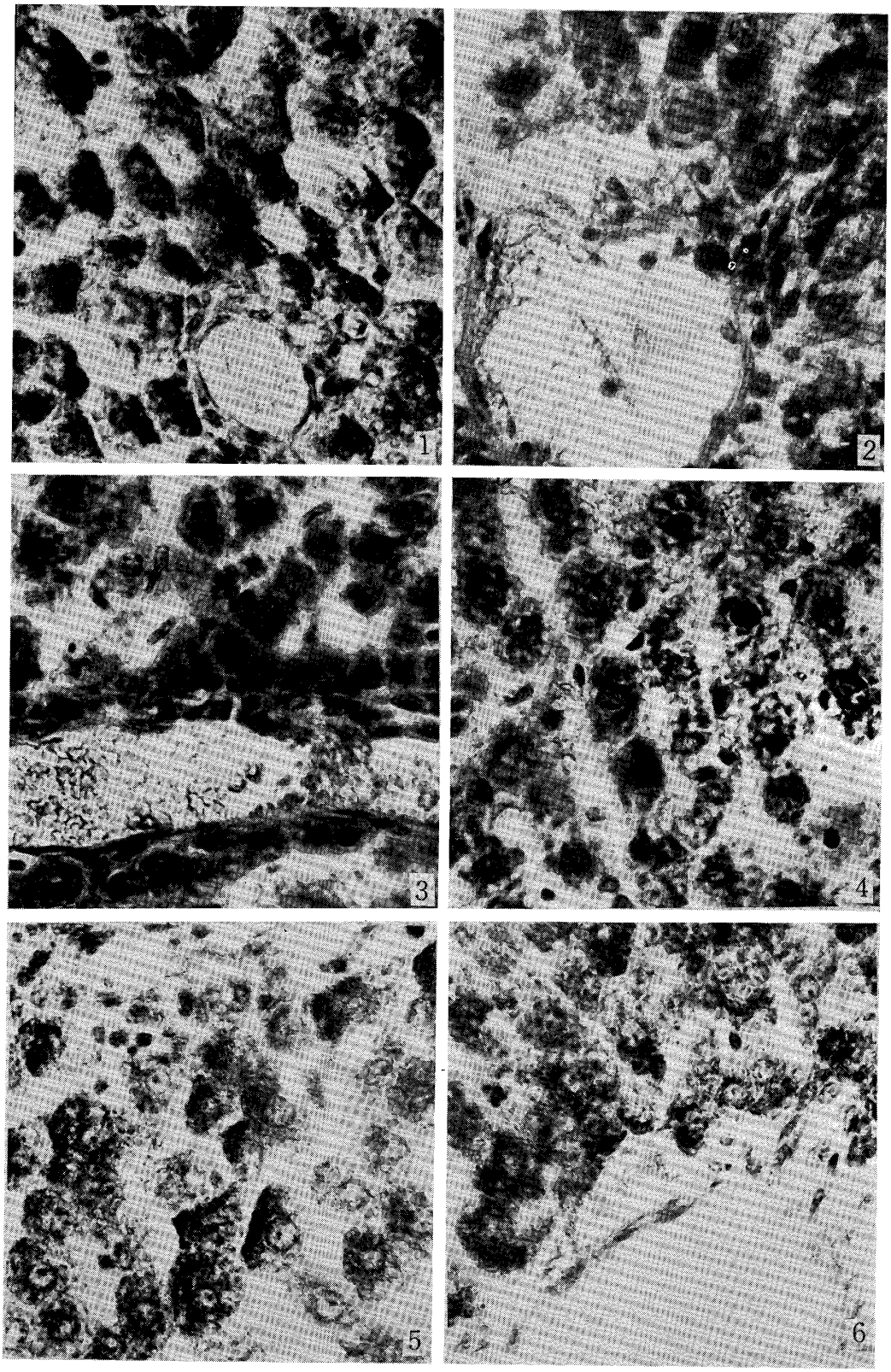
1. Fixation

Fixing fluids containing picric acid gave good results, notably Gendre's fixative for the demonstration of glycogen was best for this staining.

Gendre's fluid:

Saturated solution of picric acid in 90% ethyl alcohol	80 parts
Formaldehyde (commersial)	15 parts
Glacial acetic acid	5 parts

Materials were fixed in ice box (2–3°C) for 18 hours, and then were washed in absolute alcohol changing thrice. The addition of Li_2CO_3 was dispensable.



The fixation by formaldehyde, especially by Lilie's fluid (pH 7.0, buffered with phosphates) also gave good results.

2. Staining

After removing paraffin in benzen, the sections were stained in Ehrlich's hematoxylin (positive nuclear staining). The differentiation in acid alcohol should be avoided. After washing by running water, the glycogen was stained with Best's carmin for 20 minutes. The original concentration of Best's carmin solution was preferable. The superfluous dye solution was removed with filter paper by lightly pressing upon the sections. The sections were then differentiated in the following fluid.

Differentiation fluid:

Methyl alcohol	40 ml.
Ethyl alcohol	80 ml.
Distilled water	100 ml.

The sections were stained in the following dye solution for 13 times of to and fro swinging in the dye bottle.

Dye solution:

Fast green	0.5 g
Aurantia	0.7 g
95% ethyl alcohol	100 ml.

The superfluous dye solution was roughly removed with filter paper by pressing lightly with fingers on the sections. The preparation were then directly plunged in 95% alcohol and washed by to and fro swinging 5 times in the bottle. And then they were passed through absolute alcohol, xylol and mounted with balsam as usual. The exposure of the preparation to light should be avoided in conservation.

3. Result

Nuclei stain violet; glycogen granules red; cytoplasm light green; connective tissue fibers blue; erythrocytes yellow; and cholesterol drops orange.

4. Explanation of figures

Figs. 1-6: Gendre's fixative. In Fig. 1, artificial shift of glycogen to one side of the liver cells brought about by the infiltration of alcohol into cells is marked. The connective tissue fibers around the blood capillaries appear blue. Erythrocytes are stained yellow. Figs. 3 and 4: Lilie's fixative. Glycogen is stained less markedly. Figs. 5 and 6: Sections subjected to saliva digestion. The glycogen is roughly removed.

B. Triple staining method

1. Fixation

Almost all fixatives employed in routine works will do. Among them, those which contain picric acid, sublimate were suitable to this staining technique. To demonstrate glycogen granules, Gendre's and Lilie's gave better results as in the case of quadruple staining method.

2. Staining

After removing paraffin, the sections were transfered through alcohol series down to the distilled water. They were then stained by 0.2% cresyl violet in distilled water for 5 to 10 minutes. The superfluous dye solution was blotted with filter paper by lightly pressing with fingers upon it. The over staining with cresyl violet was differentiated by 8 times of to and fro swinging in the methyl alcohol-ethyl alcohol-distilled water mixture. The sections were then stained for 13 times of to and fro swining with the following dye solution.

Dye solution:

Fast green	0.5 g
Aurantia	0.7 g
95% ethyl alcohol	100 ml.

The superfluous dye solution was roughly removed by pressing lightly with fingers on the filter paper. The preparations were then directly plunged into 95% alcohol and washed in it by 5 times of to and fro swinging. By this procedure, some of fast green dissolved out into alcohol. And then they were soaked in absolute alcohol and xylol respectively, and were mounted by balsam as usual.

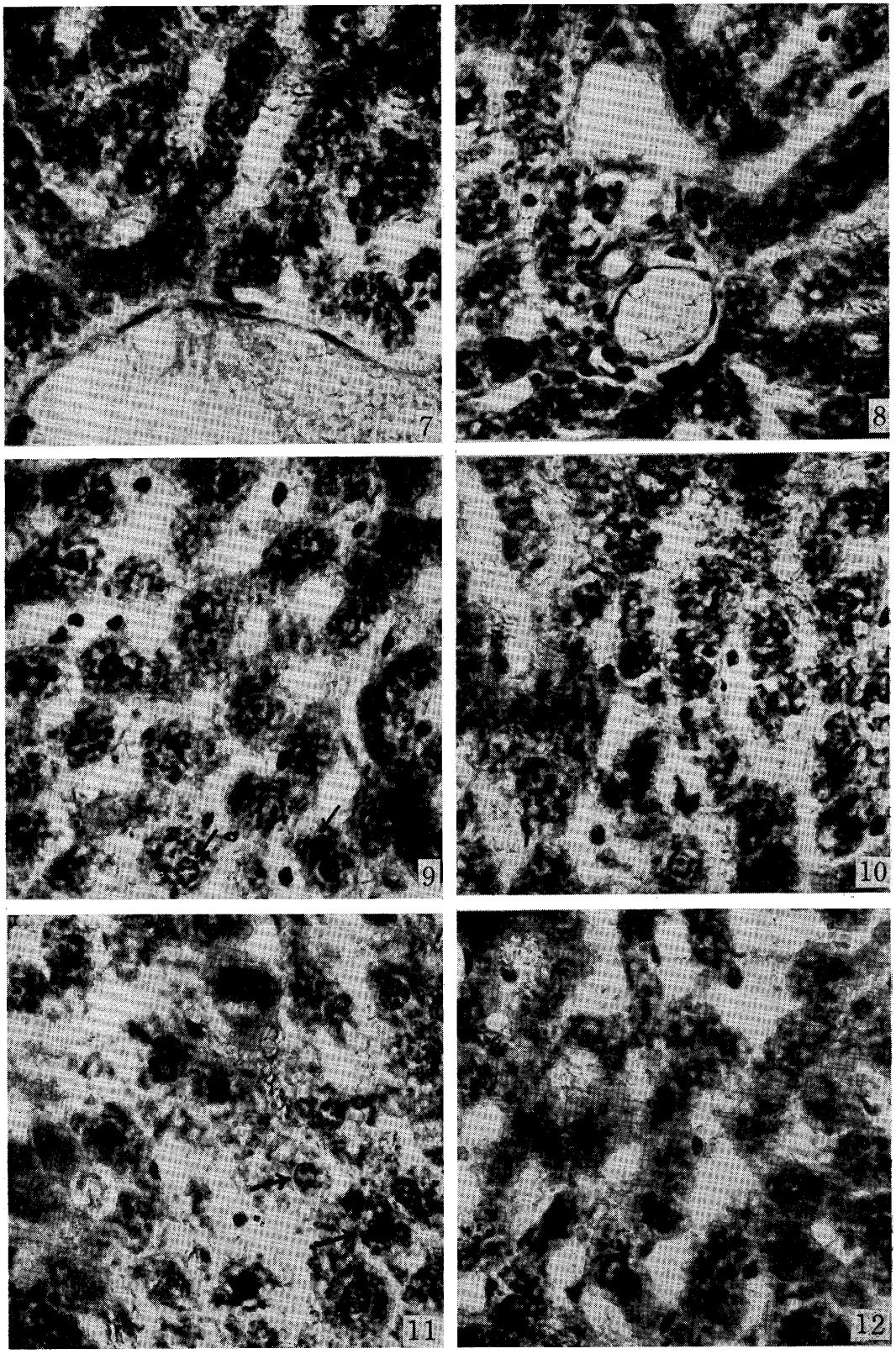
3. Result

Nuclei are stained by cresyl violet from bluish violet to red according to the unknown physiological conditions. These colour tones may possibly be of value for the conjecture of physiological milieu in cells. The cytoplasm colours lightly green. Connective tissue fibers colour blue, while muscle fibers greenish blue. Erythrocytes are stained yellow. Aurantia stains erythrocytes very intensely so that only one cell is able to be distinguishable among tissues.

4. Explanation of figures (Figs. 7–12)

The triple staining was tried upon the following kind of tissues in rat.

(a) Liver cells (Figs. 7–12) Liver is composed of liver cells, Kupffer's stellate cells, blood capillaries, connective tissue fibers, bile capillaries, and erythrocytes. Nuclei of liver cells are tinged by cresyl violet bluish violet to red according to unknown physiological condition (Figs. 9 and 11; left arrow indicates blue nuclei and right arrow red nuclei). Nuclei with intermediate tones are also observed. The cytoplasm contains always bluish violet granules which often grow into irregular masses. The nature of these masses are planning to study in future; yet they seem to be a complex of protein and glycogen. The latter are proven by the saliva test (Figs. 11 and 12). In Figs. 11 and 12, the glycogen is digested by the diastase of the saliva. The remaining substance stained blue are probably protein in nature, yet the future analysis is expected. The ground cytoplasm stains green by fast



green. The cross sections of veinous blood capillaries are seen in Figs. 7 and 8, in which the endothelial walls are clearly observable in blue

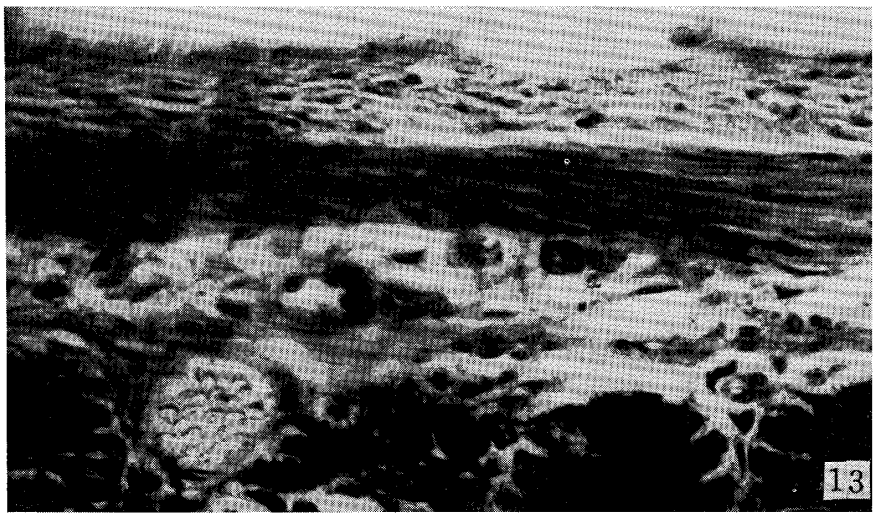
colour. These veinous capillaries and the blood sinusoids among liver cells (Figs. 7 and 8) contain erythrocytes stained yellow by aurantia. The yellow colour of erythrocytes is so intense that only a few cells in the tissue are detectable. The blood sinusoid (Figs. 7 and 8) and the bile capillaries (Figs. 9 and 10) are lined with the reticular fibers which stain green by fast green.

(b) Other organs (Figs. 13–16) Fig. 13 indicates the section of the wall of small intestine in rat. The uppermost part is the serous membrane of connective tissue which is stained blue. The next lower part is the muscular layer stained deep green. The submucosa stains blue in which the thin subepithelial muscular layer, muscularis mucosa, and a blood capillary (left lower) are embedded. The fibers of the connective tissue stain always blue, while the muscle fibers green. Thus these two components are differentiated distinctly each other. The intestinal epithelia of villi are brown by this technique (lowermost part of Fig. 13).

Fig. 14 is a photomicrograph of pancreas. Nuclei of acinar cells are all stained red (brownish), and their cytoplasm green. The parts of cytoplasm rich in endoplasmic reticulum stain blue. The islets of Langerhans (left lower part of Fig. 14) are discernible among the glandular acini by a little fainter colour from the latter. The blood capillaries are easily observed by the yellow colour of erythrocytes.

Figs. 15 and 16 are the photomicrographs of the sections of kidney in rat. Both contain glomeruli. They are each covered by Bowman's capsule (capsular endothelium) stained blue. The glomerulus is composed of glomerular epithelium which anastomoses forming a net work. In the glomeruli here and there the erythrocytes are found in yellow colour. The cells of the convoluted tubuli stain bluish green and their nuclei red or sometimes blue. The blood capillaries among the convoluted tubuli are apparent by the yellow staining of their erythrocytes (shown by arrows).

(c) Muscle Abdominal muscle in the rat was fixed with Gendre's fixative on one hand, and Lilie's on the other. The sections were stained by cresyl violet-fast green-aurantia method. Muscle fibers are coloured green, while connective tissue fibers blue. Nuclei in muscle appear reddish brown, and glycogen stains more reddish (Figs. 17 and 18). Glycogen shifts to one side of muscle fibers by the perfusion effect of the alcohol contained in Gendre's fixative. The red colour of glycogen almost disappears after treatment with saliva (30 minutes at 38°C) (Fig. 19). In the sections fixed with Lilie's fixative, glycogen in the muscle fiber was found in diffuse violet by cresyl violet. Glycogen preserved by Lilie's fluid was also lost by the digestion with saliva (Fig. 20). The above stated results were also ascertained by PAS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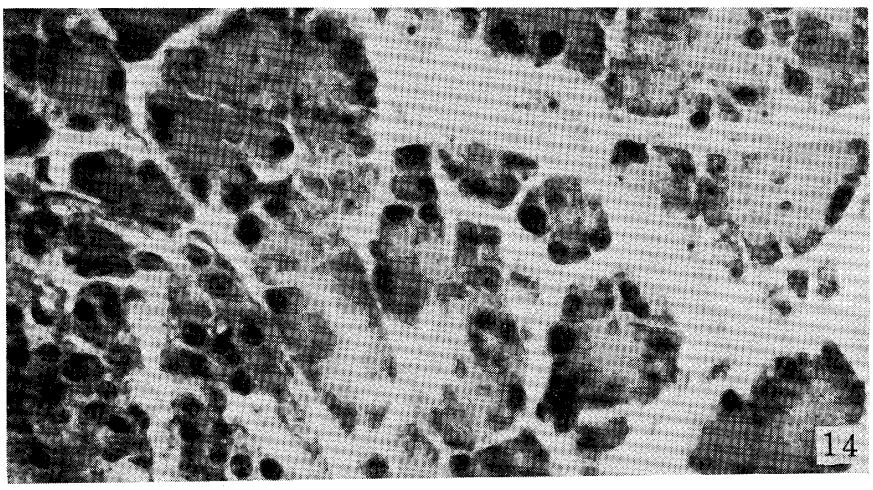
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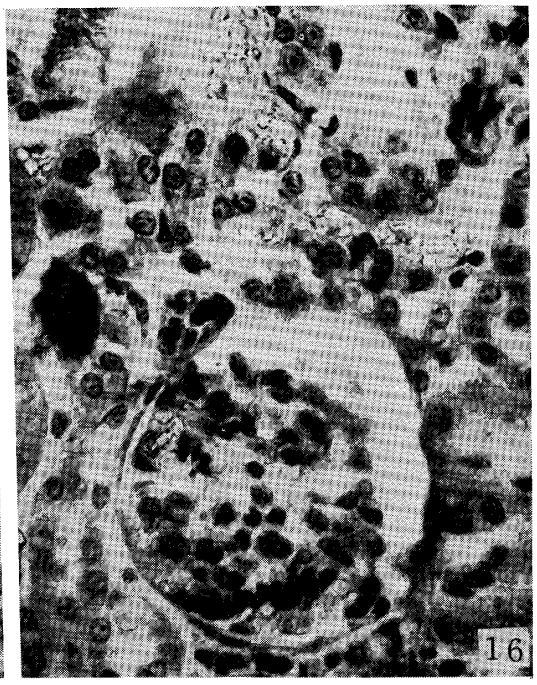
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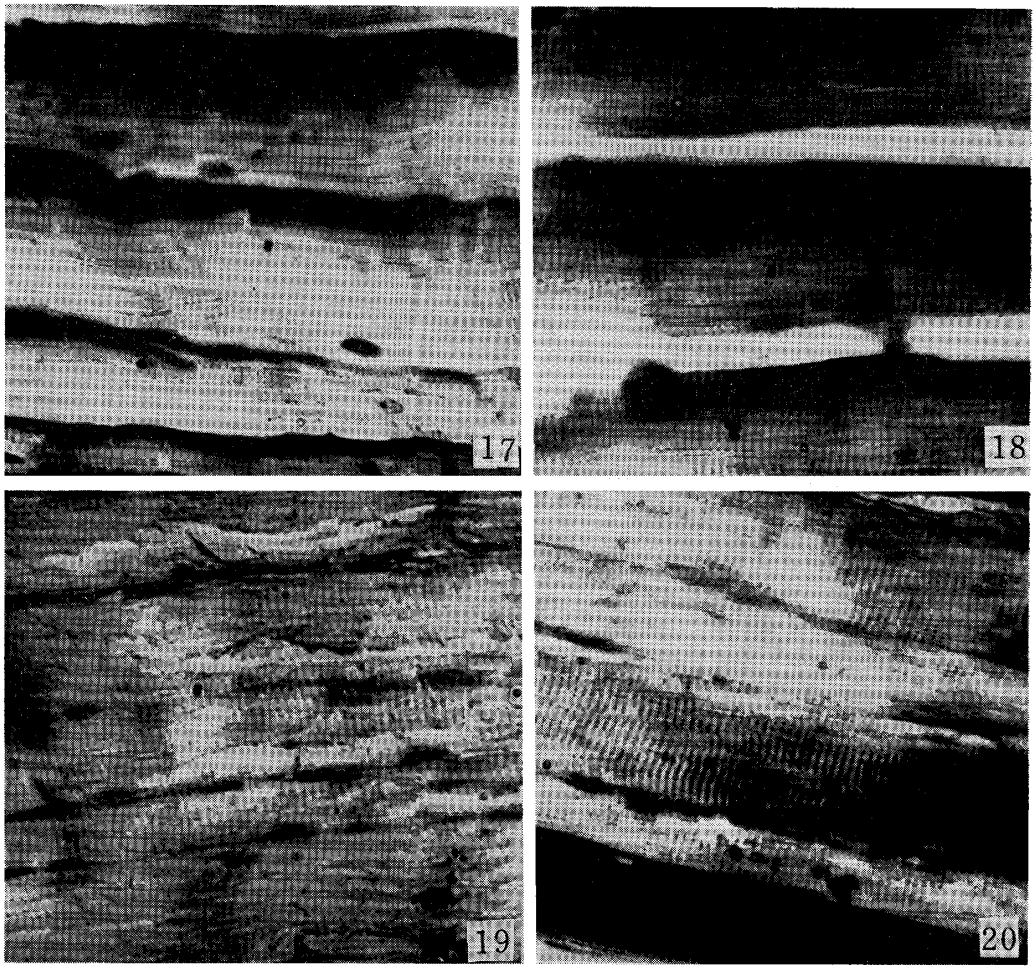

method. Results got by the new technique and those by PAS method went quite parallel with each other. After the digestion with saliva, glycogen still remains to a certain amount. This kind of glycogen component is supposed to be the so-called desmoglycogen, which stains pink with cresyl violet and PAS (see Pearse, 1954).

Critique of new Method

Both methods (quadruple and triple staining methods) are based upon the staining with hematoxylin or cresyl violet, fast geen and aurantia. The combination with other specific cytochemistry is probable; for example, with Best's carmin method or PAS method etc. Of these above stated two techniques the cresyl violet-fast green-aurantia combination is excellent for the non-specific staining of the tissues. It gives a brilliant contrast in colours. Especially, the erythrocytes are yellow among other tissue elements. Although a brilliant staining is obtainable, the exposure to light in a long period should be avoided, otherwise the green colour fades gradually. Yet this fading of green colour of fast green is by far slower than in the case of staining with

light green (see Conn, 1936). Nuclei are stained from blue to red according to the unknown physiological conditions. The cause of this phenomenon should be studied in future.

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